Biochimica et Biophysica Acta 1798 (2010) 1008–1013

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Sterol affinity for bilayer membranes is affected by their ceramide content and the ceramide chain length

Thomas K.M. Nyholm, Pia-Maria Grandell, Bodil Westerlund, J. Peter Slotte *

Åbo Akademi University, Department of biochemistry and pharmacy, Tykistökatu 6A, 20520 Turku, Finland

ARTICLE INFO

Article history:
Received 10 November 2009
Received in revised form 22 December 2009
Accepted 24 December 2009
Available online 4 January 2010

Keywords:
Dioleoyl phosphatidylcholine
Palmitoyl sphingomyelin
Cholesterol
Cholestatrienol
Lateral domain
Membrane structure

ABSTRACT

It is known that ceramides can influence the lateral organization in biological membranes. In particular ceramides have been shown to alter the composition of cholesterol and sphingolipid enriched nanoscopic domains, by displacing cholesterol, and forming gel phase domains with sphingomyelin. Here we have investigated how the bilayer content of ceramides and their chain length influence sterol partitioning into the membranes. The effect of ceramides with saturated chains ranging from 4 to 24 carbons in length was investigated. In addition, unsaturated 18:1- and 24:1-ceramides were also examined. The sterol partitioning into bilayer membranes was studied by measuring the distribution of cholestatrienol, a fluorescent cholesterol analogue, between methyl-β-cyclodextrin and large unilamellar vesicle with defined lipid composition. Up to 15 mol% ceramide was added to bilayers composed of DOPC:PSM:cholesterol (3:1:1), and the effect on sterol partitioning was measured. Both at 23 and 37 °C addition of ceramide affected the sterol partitioning in a chain length dependent manner, so that the ceramides with intermediate chain lengths were the most effective in reducing sterol partitioning into the membranes. At 23 °C the 18:1-ceramide was not as effective at inhibiting sterol partitioning into the vesicles as its saturated equivalent, but at 37 °C the additional double bond had no effect. The longer 24:1-ceramide behaved as 24:0-ceramide at both temperatures. In conclusion, this work shows how the distribution of sterols within sphingomyelin-containing membranes is affected by the acyl chain composition in ceramides. The overall membrane partitioning measured in this study reflects the differential partitioning of sterol into ordered domains where ceramides compete with the sterol for association with sphingomyelin.

1. Introduction

Sphingomyelin is a major phospholipid in cell membranes of many eukaryotic cells, and is known to have a major impact on the biophysical properties of membrane bilayers [1–3]. Sphingomyelin together with membrane cholesterol form ordered domains, and in cell membranes such domains are termed lipid rafts[4,5]. In addition to containing sphingomyelin and cholesterol, lipid rafts also invariably contain other ordered lipids such as glycosphingolipids. The rafts are believed to be important transient platforms for cell signaling and have been suggested to also take part in membrane trafficking and protein sorting [4,6–9].

The level of cell membrane sphingomyelin is in part regulated by the activity of endogenous or exogenous sphingomyelinases, yielding ceramide as the immediate product of the catalytic reaction[10]. The properties of the formed ceramide-enriched domains or platforms are largely dependent on the physical properties of the ceramide species found in the domains. As in most biological sphingolipids, the acyl chains of ceramides mainly vary from 14 to 24 carbons. Ceramides are at least partly miscible with sphingomyelin and longer-chain ceramides have been shown to partition favorably into sphingomyelin-rich domains [11–15]. Work with model membrane systems [16–18], lipoproteins [19], and caveolin-rich lipid rafts [20] has revealed that certain ceramides are able to displace cholesterol from sphingomyelin-rich or saturated phosphatidylcholine-rich domains. It was also shown that the partitioning of longer-chain ceramides into the sphingomyelin-rich domains (from which cholesterol was displaced) resulted in a marked stabilization of the ceramide/sphingomyelin domains against temperature-induced melting [18]. These findings imply that longer-chain ceramides have a more favored interaction with ordered phases as compared to cholesterol, or conversely that such ceramides avoid disordered phases more than cholesterol. We have showed how the interaction of ceramides with other lipids in bilayers composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), D-*erythro*-*N*-palmitoyl sphingomyelin (PSM) and cholesterol was chain length dependent [21].

A commonly used approach to investigate cholesterol interactions with phospholipids has been to measure sterol partitioning between

Abbreviations: CTL, cholesta-5,7,9(11)-trien-3-beta-ol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PCer, D-*erythro*-*N*-palmitoyl ceramide; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSM, D-*erythro*-*N*-palmitoyl sphingomyelin

* Corresponding author.
E-mail address: jpslotte@abo.fi (J.P. Slotte).

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2009.12.025

methyl-β-cyclodextrin (mβCD) and phospholipid bilayers [22–25], or sterol efflux to mβCD from phospholipid monolayers [26]. This kind of experiment has been used to determine the affinity of cholesterol for bilayers composed of different phospholipids, and it has been shown that the measured affinities also reflect how cholesterol is laterally distributed in phospholipid bilayers [27]. Hence, data obtained on cholesterol partitioning between mβCD and phospholipid bilayers can be used to evaluate how cholesterol and phospholipid may organize laterally in biological membranes.

In the present study we have further investigated how the membrane concentration of ceramide and how the ceramide molecular species influence the partitioning of sterol within phospholipid bilayers. We examined the equilibrium partitioning of cholestatrienol (CTL) between bilayer membranes, having different lipid and ceramide compositions, and mβCD [24]. We find that CTL (and cholesterol) membrane partition was reduced in a linear fashion as the membrane content of ceramide is increased (up to 15 mol%). Saturated ceramides having chain lengths between 12 and 18 were the most effective in reducing sterol partitioning. This study conclusively shows a direct relationship between the membrane ceramide content and sterol partitioning into membranes. Thus ceramide is a potent regulator of sterol distribution among membranes. This effect of ceramide is likely to be of high biological significance.

2. Materials and methods

2.1. Materials

PSM was purified from egg sphingomyelin (Avanti Polar Lipids, Alabaster, AL) by reverse-phase HPLC (Supelco Discovery C18-column, dimensions 250×21.2 mm, 5μm particle size) using 100% methanol as eluent. The purity and identity of the product was positively verified by ESI-MS. Acyl-chain defined ceramides were obtained from Avanti Polar Lipids. DOPC was obtained from Avanti Polar Lipids and cholesterol was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). CTL was synthesized and purified as described previously [28]. CTL was stored dry under argon at −86 °C. Stock solutions of CTL were prepared in argon-purged methanol and were used within a few days (storage −20 °C). Sphingomyelin and ceramide stock solutions were prepared in hexane/isopropanol (3:2, by vol). Solutions were stored in the dark at −20 °C, and warmed to ambient temperature before use. The water used for all experiments was purified by reverse osmosis, followed by passage through a Millipore UF Plus water-purification system (Millipore, Billerica, MA), to yield a product resistivity of 18.2 MΩcm.

2.2. Sterol partitioning into membrane bilayers

The distribution of CTL between mβCD (Sigma Chemicals, St. Louis, MO) and large unilamellar phospholipid vesicles was determined as described by (Nyholm et al., in preparation 2009), a method significantly modified from the procedure reported by Niu and Litman [24]. All lipids including CTL were mixed in chloroform after which the solvent was evaporated. The dry lipid was then rehydrated at 65 °C and vortexed vigorously. The resulting multi lamellar vesicles were then filtered 12 times through a filter with a pore size of 200 nm (Whatman International, Maidstone, UK) at 65 °C, giving large unilamellar vesicles (LUVs) with a diameter of about 200 nm. For the partitioning assay 100 nmol lipids (LUVs) were portioned into 10 glass tubes. mβCD was added to nine of the tubes, after which the solutions were diluted with milli-Q water to a final phospholipid concentration of 40 μM. The final concentration of mβCD in the tubes was 0, 0.04, 0.08, 0.15, 0.25, 0.35, 0.50, 0.60, 0.80, and 1.0 mM. All samples were incubated over night at 23 °C or for 2 h at 37 °C to achieve equilibrium partitioning of CTL between mβCD and LUVs. The steady state anisotropy of CTL was measured in PTI Quantamaster 1 (Photon Technology International Inc., New Jersey, USA) spectrofluorimeter operating in the T-format, with both the excitation and emission slits set to 5 nm. The samples were excited at 324 nm and the emission was measured at 390 nm.

The molar concentration of CTL (C_{CTL}^{LUV}) in the LUVs in each sample was calculated from the measured anisotropy according to

$$C_{CTL}^{LUV} = C_{CTL}\frac{(r_i - r_{CD})}{(r_{LUV} - r_{CD})} \quad (1)$$

where C_{CTL} is the total concentration of CTL in the samples, r_{LUV} is the anisotropy of CTL in the specific phospholipid bilayer, r_i is the CTL anisotropy in the sample and r_{CD} is the anisotropy of CTL in the CTL–mβCD complex. The anisotropy of the CTL–mβCD complex was measured for a range of the CTL–mβCD ratios and was determined to 0.175 at 23 °C and 0.170 at 37 °C.

The molar fraction partition coefficient K_X was calculated as described by Tsamaloukas et al. [25] based on the equation

$$K_x = \frac{C_{CTL}^{LUV}(C_{CD})^n}{(C_L + C_{CTL}^{LUV})C_{CTL}^{CD}} \quad (2)$$

where C_L is the phospholipid concentration, C_{CD} is the cyclodextrin concentration, C_{CHOL}^{LUV} is the cholesterol concentration in lipid bilayers and C_{CHOL}^{CD} is the concentration of cholesterol in complex with mβCD. The partition coefficient was calculated by plotting the calculated molar concentrations of CTL in the phospholipid bilayers against the mβCD concentration and fitting the obtained curves with the following equation.

$$C_{CTL}^{LUV} = \frac{C_L - C_{CTL} + (C_{CD})^n/K_x}{2} \times \left(\sqrt{1 + 4\frac{C_L C_{CTL}}{[C_L - C_{CTL} + (C_{CD})^n/K_x]^2}} - 1\right). \quad (3)$$

The stoichiometry in the cholesterol:mβCD complexes was assumed to be 1:2 as reported by Tsamaloukas et al. [25].

3. Results

3.1. Partitioning of CTL between mβCD and unilamellar phospholipid vesicles

The recently developed CTL equilibrium partition assay has been used to determine the affinity of sterols for phospholipid bilayers (Nyholm et al., in preparation 2009). To illustrate the CTL partition method used, we show representative data in Fig. 1. Unilamellar vesicles of DOPC with 2 mol% CTL were incubated with an increasing amount of mβCD and the steady state anisotropy was measured at 23 °C. In addition, the anisotropy of CTL in mβCD complexes was measured at the same temperature. CTL in mβCD in the absence of bilayer membranes gave a fairly low anisotropy (~0.175). The anisotropy of CTL in bilayers was much higher (~0.255; Fig. 1A), but as the mβCD concentration increased the anisotropy was lowered towards that of CTL–mβCD complexes. As the fluorescence intensity of CTL did not change significantly during the various conditions used, the concentration of CTL in DOPC bilayers could be calculated from the anisotropy data using Eq. (1). Fitting the anisotropy versus mβCD function using Eq. (3) yields the CTL partition coefficient (assuming 1:2 CTL: mβCD ratio; Fig. 1B). The quality of the fit was usually very good (R^2>0.98).

Since PSM interacts favorably with cholesterol (and other sterols), a good validation of the CTL partition assay would be to determine K_x in e.g., DOPC bilayers as a function of increasing the PSM content in the membrane. The partitioning of cholesterol into unsaturated phospholipid bilayers is known to be much lower compared to bilayers which also contain saturated phospholipids [24,29–31]. Fig. 2 shows our results for the partitioning of CTL into DOPC bilayers as the

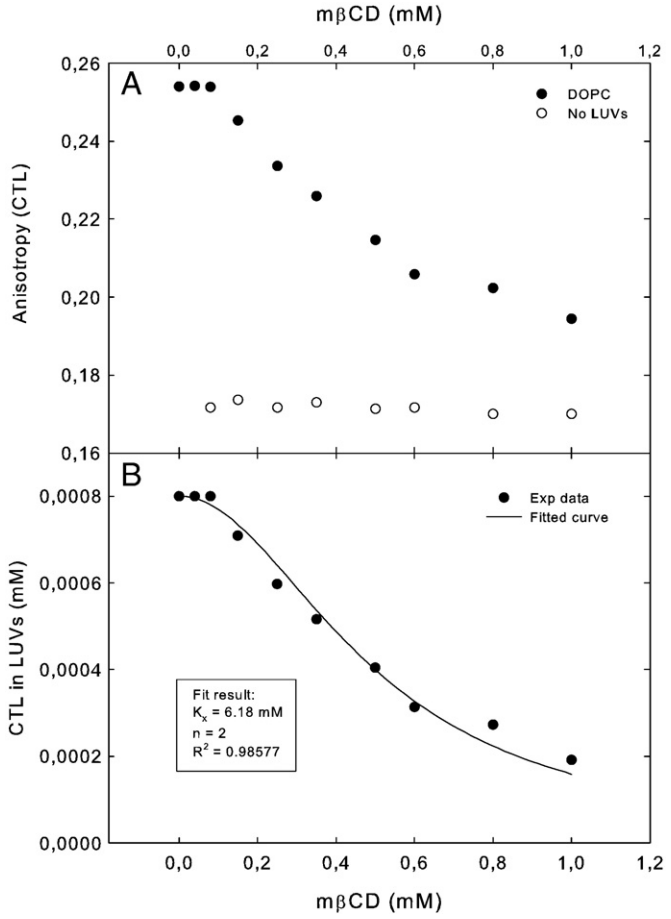

Fig. 1. Determination of liposome CTL partition coefficients, based on CTL anisotropy measurement at equilibrium. Panel A shows CTL anisotropy measurements in DOPC bilayer samples with increasing mβCD concentration (cf. the experimental section), and panel B gives the fitted function that best described the anisotropy data with the corresponding molar partition coefficient (K_x) for CTL.

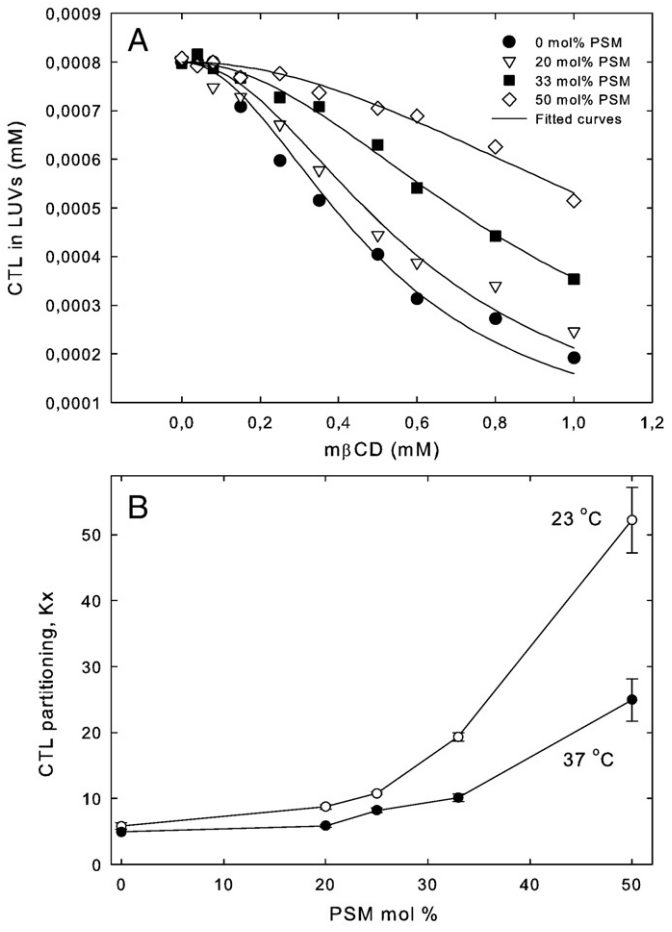

Fig. 2. Partitioning of CTL into DOPC bilayers as a function of increasing PSM concentration in the bilayers. Equilibrium partitioning was performed at 23 °C, and the CTL anisotropy as a function of mβCD was fitted (panel A) to yield the K_x (panel B). Each value in panel B is the average from three separate experiments ± SD.

PSM concentration varied between 0 and 50 mol%. Panel A of Fig. 2 shows the fitted curves indicating the distribution of CTL between bilayer membranes and mβCD for varying PSM concentration at 23 °C. As the PSM content increased, the fraction of CTL present in bilayers increased. The calculated K_x for CTL at both 23 and 37 °C is shown in panel B of Fig. 2. The affinity of CTL for the bilayer was higher at the lower temperature, and markedly so when gel phase PSM was present in the bilayers (above 30 mol% at 23 °C [32]). These data demonstrate clearly the fact that a high K_x is indicative of favorable partitioning into the membrane relative to mβCD. The absolute partition coefficients were lower for CTL than for cholesterol but the addition of 50 mol% PSM increased K_x similarly as has been observed with cholesterol [23]. Hence, we consider CTL to mirror very well the partition behavior of cholesterol.

3.2. Ceramide affected sterol partitioning between mβCD and unilamellar vesicles

Since the aim of the project was to study the effects of ceramides on sterol partitioning into bilayer membranes, we wanted to first test the effects of PCer on sterol free bilayer membranes (save for the 2 mol% CTL always present in the system). In DOPC bilayers containing no saturated phospholipid, the increasing presence of PCer had no significant effect on the partitioning of CTL between mβCD and the bilayer membranes at 23 °C (Fig. 3A) or 37 °C (Fig. 3B). We also observed that another ceramide, i.e., oleoyl ceramide, at 7.5 and 15 mol% failed to affect CTL partitioning (data not shown). However, when PSM was present (DOPC:PSM 4:1 by mol), the K_x of CTL was higher as compared with PSM-free DOPC bilayers (e.g., at 0 mol% PCer), both at 23 and 37 °C (Fig. 3A and B, respectively). This finding was fully expected since PSM is known to increase the partition coefficient of cholesterol (and CTL) for unsaturated phospholipid bilayers [23]. In the DOPC:PSM bilayer system, the increased presence of PCer led to a linear decrease in the K_x of CTL (Fig. 3). This decrease was more pronounced at 23 than at 37 °C. The conclusions from these experiments were that whereas PCer did not affect partitioning of CTL in totally fluid membranes, it did so in fluid membranes that also included a saturated phospholipid.

Next we wanted to determine the effects of PCer on CTL partitioning in ternary bilayer membranes containing DOPC, PSM and cholesterol (3:1:1 by mol). This composition has previously been shown to have fluid–fluid phase coexistence at 23 °C but not at 37 °C [32]. In line with this the presence of 20 mol% cholesterol increased K_x more at 23 than at 37 °C.

The increased membrane content of PCer led to a dramatic decrease in CTL partitioning into the membrane (Fig. 4). Again the effect was more pronounced at 23 than 37 °C, but the effect of PCer was clearly seen even at 37 °C. The effect seen at 23 °C was possibly due to the presence of liquid ordered domains at that temperature. In previous studies it has been shown that PCer can displace cholesterol or CTL from ordered domains [16,18,20,33], but our results now show that the overall partitioning of the sterol into the PSM-containing membrane is affected negatively by the presence of PCer.

Since the ceramide chain length is known to affect the efficacy of the molecule to interfere with sterol distribution in membranes [21,34], we also wanted to examine how different ceramide species were able to

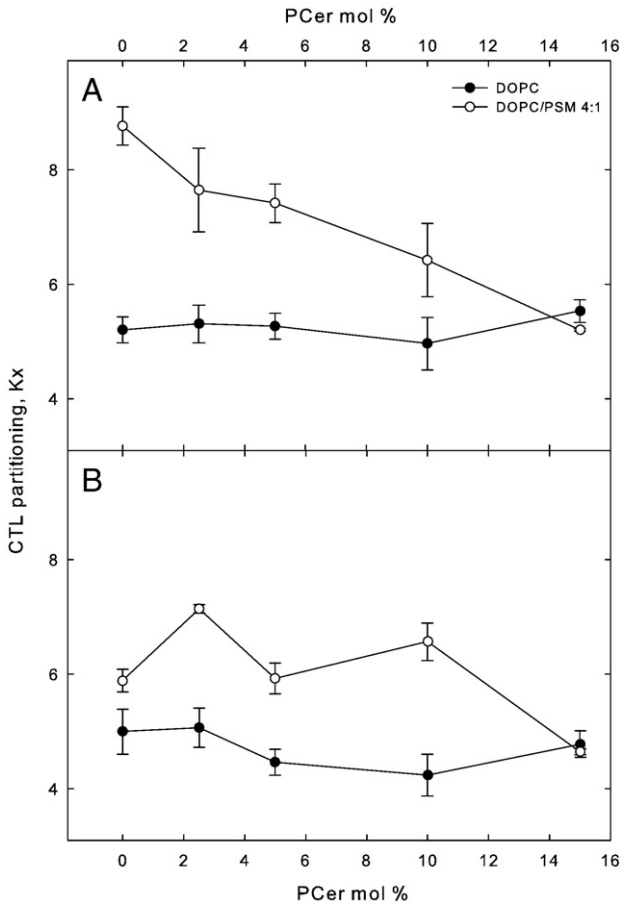

Fig. 3. Partitioning of CTL into DOPC or DOPC:PSM bilayers as a function of increasing PCer concentration in the bilayers. CTL partitioning was determined as described in the Materials and methods section. The initial bilayer composition was pure DOPC or DOPC:PSM (4:1 by mol) and into this PCer was added at the indicated concentrations (mol% of the total lipid). Panel A depicts partitioning at 23 °C and panel B at 37 °C. Each value is the average from three separate experiments ± S.D.

affect CTL partitioning in ternary bilayer membranes containing DOPC:PSM:cholesterol (3:1:1 by mol). The CTL K_x plotted as a function of the ceramide species is shown in Fig. 5. All the ceramides had sphingosine as the long-chain base and only varied with regard to the *N*-linked fatty acid. C4:0-ceramide at 15 mol% did not affect CTL partitioning at all (neither at 23 nor 37 °C, Fig. 5A and B). All other longer-chain ceramides tested caused a decrease in the K_x for CTL, with C12:0, C16:0 (PCer) and C18:0 ceramides being the most effective (at 23 °C). At 23 °C the 18:1$^{\Delta 9}$ ceramide reduced CTL partitioning, but not as efficiently as 18:0 ceramide. At 37 °C this difference between 18:0 and 18:1 ceramide was no longer seen. The 24:1$^{\Delta 15}$ ceramide behaved like the 24:0 ceramide with regard to affecting the CTL partitioning (at both temperatures tested, Fig. 5A and B).

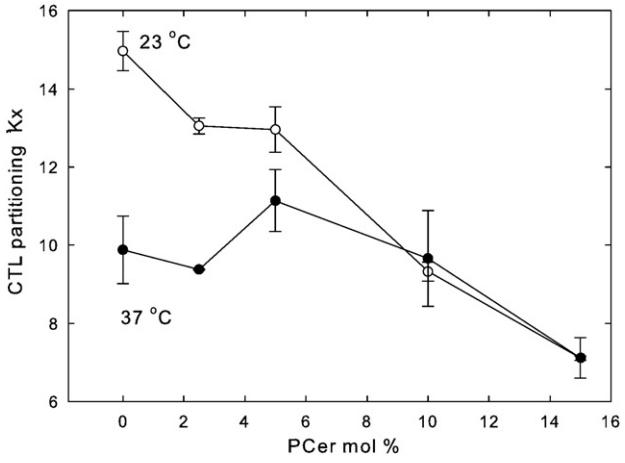

Fig. 4. Partitioning of CTL into DOPC:PSM:cholesterol bilayers as a function of increasing PCer concentration in the bilayers. CTL partitioning was determined as described in the Materials and methods section. The initial bilayer composition was DOPC:PSM:cholesterol 3:1:1 by mol and into this PCer was added at the indicated concentrations (mol% of the total lipid). Each value is the average from three separate experiments ± S.D.

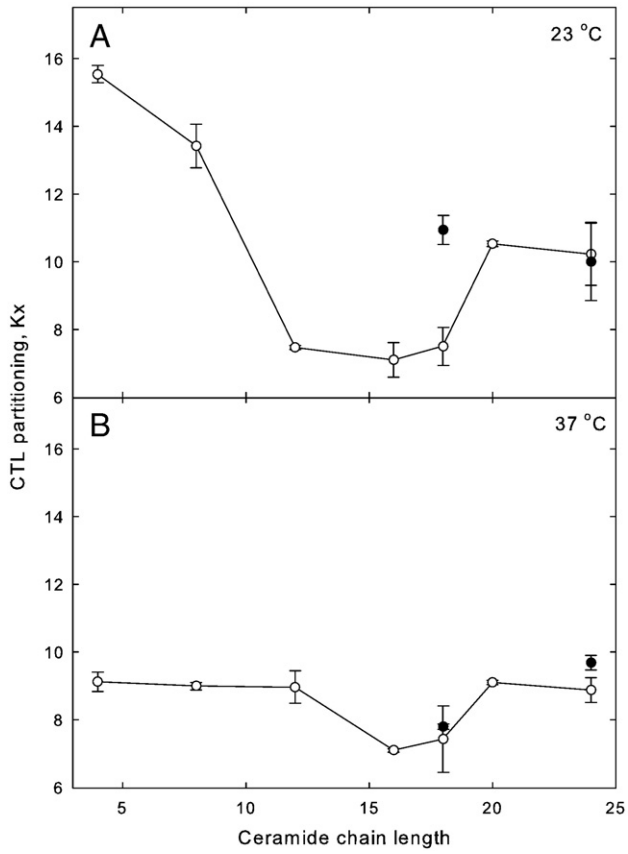

Fig. 5. Partitioning of CTL into DOPC:PSM:cholesterol:ceramide bilayers as a function of ceramide chain length. CTL partitioning was determined as described in the experimental section. The bilayer composition was DOPC:PSM:cholesterol 3:1:1 with ceramide added as 15 mol%. The ceramide *N*-linked acyl chain varied between 4 and 24 carbons and was either saturated (open symbols) or monounsaturated (18:1$^{\Delta 9}$ or 24:1$^{\Delta 15}$; closed symbols). Panel A depicts partition at 23 °C and panel B at 37 °C. Each value is the average from three separate experiments ± S.D.

4. Discussion

The lateral distribution of cholesterol in a membrane is determined by the components of the membrane, and this lateral distribution is in part related to the chemical potential of cholesterol. When cholesterol is interacting favorably with a phospholipid (e.g. with saturated SM) its chemical potential is lower compared to a situation where it interacts unfavorably with a phospholipid (e.g. DOPC). This change in chemical potential of cholesterol can be determined from a change in cholesterol oxidation susceptibility [35] or from its membrane escape fugacity [23,24,26,35]. In this study, the changes in chemical potential of cholesterol in membranes were determined as a function of the membrane ceramide composition. The chemical potential of CTL (and indirectly of cholesterol) was determined by measuring the equilibrium partitioning coefficient, which relates to the membrane escape fugacity of the sterol.

Previously sterol partitioning experiments have been performed using mβCD and [^{3}H]cholesterol [24]. The drawback of the method was

that the cholesterol-mβCD complexes had to be separated from the lipid vesicles in order to determine the fraction of cholesterol in vesicles and mβCD, respectively. The separation method could possibly shift the equilibrium and hence the calculated partition coefficient. Another approach that has been used is isothermal titration calorimetry [25]. Although this is an excellent method to determine sterol partition coefficients it also has drawbacks. First, the amount of material that has to be used is large compared to fluorescence experiments. Second, to keep the total lipid concentrations as low as possible, high concentrations of cholesterol has to be used. With our approach to use CTL, we can measure partition coefficients with low concentrations of lipids, and at sterol concentrations were phase separation induced by the sterol is not expected to happen. Further, separation of membrane- and mβCD-bound CTL is not necessary. A possible drawback of studying a sterol analogue instead of cholesterol is that the two may not always behave similarly. In fact, all partition coefficients measured with CTL are lower than those for cholesterol. For example with pure DOPC bilayers the K_x is about 6 mM for CTL at 23 °C (Fig. 1), and about 5 mM at 37 °C. For cholesterol in pure DOPC at 37 °C a K_x of about 47 mM has been reported [24]. We think this difference in K_x is in part due to the more polar nature of CTL, and to the fact that CTL and cholesterol are expected to have different affinities for mβCD. To be able to compare CTL and cholesterol interactions with phospholipids we measured CTL partitioning in DOPC bilayers with an increasing amount of PSM (Fig. 2). If the partitioning behavior of CTL responds similarly to changes in lipid bilayer composition as that of cholesterol, the sterols interact similarly (relatively speaking) with the phospholipids. In DOPC/PSM (1:1) the K_x for CTL was about 52 mM at 37 °C, giving a relative partition coefficient (K_R) of ~5 for partitioning between DOPC/PSM (1:1) and DOPC. This is similar to what was obtained for CTL partitioning between POPC/PSM (1:1) and pure POPC (K_R ~6, Nyholm et al., in preparation 2009). From ITC studies the K_R was 4.3 for cholesterol partitioning between POPC/PSM (1:1) and POPC bilayers at 37 °C [36]. This is slightly lower than for CTL but in the ITC study the bilayers contained 20 mol% cholesterol, and K_R is known to decrease with increasing cholesterol concentration. Therefore, we conclude that CTL interacted similarly as cholesterol with the phospholipids.

Ceramides have been shown to displace cholesterol from ordered domains formed by saturated sphingomyelins or phosphatidylcholines [16,18,33]. The effect of ceramide on cholesterol displacement from ordered domains was also shown to be acyl-chain dependent, in that the chain length had to be 8 carbons or longer for cholesterol displacement to take place [21]. Shorter-chain ceramides disordered the ordered domains (save for C2 ceramide [21]), but failed to displace cholesterol. In the present study, PCer at concentrations up to 15 mol% failed to affect CTL partitioning into DOPC bilayers (Fig. 3). These findings were expected, since sterol (and ceramide) interaction with DOPC is highly unfavorable because of the disordered nature of the phospholipid. At 15 mol% PCer most likely segregated to its own phase, with which CTL failed to interact. At the low CTL concentration (max 2 mol% in the bilayer), and with the highly disordered phospholipid, no sterol-rich liquid ordered phase could be formed. The sterol was still able to coexist with DOPC in the disordered phase at that low concentration. However, when PSM was included in the DOPC bilayer at 20 mol% (Fig. 3), PCer was able to affect the partitioning of CTL in a concentration-dependent manner. Both the sterol and PCer competed for association with the large head-group PSM, but the increased presence of PCer eventually displaced CTL from the PSM environment and forced CTL to partition into the DOPC-rich disordered phase. Clearly PCer has a higher affinity for interacting with PSM as compared with CTL. PCer probably formed a gel phase with PSM in which CTL was not included. At 15 mol% PCer the K_x of CTL was at the level of pure DOPC, suggesting that at this PCer concentration most of the bilayer PSM was bound in the gel phase and unable to interact with CTL. The effect of PCer on CTL partitioning was more pronounced at 23 °C but in cholesterol and PSM containing membranes the effect was clear even at 37 °C (Fig. 4). With 20 mol% cholesterol in the bilayers the effect of PCer on K_x at 23 °C was larger than without cholesterol. This is likely because CTL is in a liquid ordered phase in the DOPC/PSM/CHOL (60:20:20) bilayers [32]. Addition of 20 mol% cholesterol to DOPC bilayers did not affect CTL partitioning between mβCD and vesicles (data not shown). These results show clearly that the lateral displacement of cholesterol, which previously was shown to be affected by PCer [16,18,33], also leads to a marked reduction in the membrane partitioning of CTL. Hence the lateral distribution of sterols in a membrane is reflected by its partitioning coefficient as determined by e.g. cyclodextrins. This relationship between lateral distribution and membrane partitioning for sterols was recently demonstrated in a different but related model membrane system [27].

The chain length dependence of the ceramide effect on CTL partitioning was examined at 15 mol% concentration in DOPC:PSM:cholesterol (3:1:1) bilayers. The C4:0 ceramide analogue had no effect on CTL partitioning (Fig. 5 versus Fig. 4 at 0 mol%), in good agreement with data from our laboratory that C4:0 ceramide was unable to displace cholesterol from PSM-rich domains , and instead destabilized PSM domains [21]. C8:0 ceramide was the shortest chain analogue that displaced cholesterol [21]. In the present study C8:0 was shown to decrease the K_x of CTL at 23 °C but not at 37 °C. Partition of the C8:0 ceramide into l_o domains at 23 °C would explain its effect on CTL partitioning at that temperature, and the absence of l_o domains at 37 °C would also be consistence with the lack of an effect on the partitioning. The longer the chain length became the more marked was the effect of the ceramide on CTL partitioning even at 37 °C. Maximal effect was seen with C16:0 and C18:0 ceramide species. These results agree with previous ceramide displacement studies [16,18,33,34]. The very long-chain ceramides (C20:0 and C24:0) were less effective than C16:0 or C18:0 ceramides in affecting the K_x of CTL. This may relate in part to the formation of high-temperature melting gel phases that failed to efficiently affect sterol/PSM interaction in the liquid phase. Of the unsaturated ceramides C24:$1^{\Delta 15}$ affected CTL partitioning to a similar extent as the 24:0 analogue, suggesting that the Δ^{c15} unsaturation did not further impair the effect of the ceramide. Long-chain ceramides (e.g., nervonoyl ceramide) are known to form interdigitated phases in fluid phosphatidylcholine bilayers [37]. It is possible that the membrane effects of some of the highly asymmetric ceramides tested in our study were influenced by the formation of such interdigitated phases.

The C18:1 ceramide was surprisingly effective in reducing the partitioning of CTL, suggesting it also displaced sterol from PSM-rich domains. Megha and London reported only a weak displacement of dehydroergosterol by C18:$1^{\Delta 9}$ ceramide relative to effect seen with the C18:0 analogue [16]. The method used to evaluate displacement is different in this study compared to that of Megha and London, although both use a fluorescent sterol (dehydroergosterol vs CTL). Despite the different techniques used to determine ceramide effects on sterol distribution, our results and those of Megha and London are strikingly compatible.

Our finding that even shorter-chain ceramides affected CTL partitioning, even though they do not induce gel phase formation , is compatible with the results presented by Chiantia et al. showing that shorter-chain ceramides partition into the l_o-phase and affect lipid interaction in that phase [38]. Megha et al. also presented evidence that shorter-chain ceramides (C2:0–C8:0) destabilize ordered domains in DOPC:PSM (1:1) bilayers, whereas longer-chain ceramides stabilized ordered domains (against temperature-induced melting [34]). Therefore it appears that ceramides can increase the chemical potential of sterols either by displacing them from a gel phase, which longer-chain ceramides can form with PSM, or by interfering with sterol/PSM interaction in the l_o-phase (without forming a gel phase). In both cases the lateral distribution of the sterol is changed, and the membrane escape fugacity is increased.

The recent findings that ceramides so dramatically affect sterol distribution in and out of membranes is further illuminated by the

observations that membranes have a limited solubility of both sterol and ceramide, and that both molecules compete for interaction with large head-group phospholipids [39]. A large head group (e.g., phosphocholine) is required for a high membrane solubility of either sterol or ceramide, since it provides good shielding against water exposure at the interface (the umbrella model [40,41]). Ali et al. also showed that long-chain ceramides appeared to interact more favorably with POPC than cholesterol did [39], a finding that also is likely to directly affect the membrane escape fugacity of sterols. In a very recent report, Castro et al. observed that ceramide gel domains which were formed in the absence of sphingomyelin could be solubilized by cholesterol [42]. It was also shown that ceramide was fairly soluble in cholesterol-rich phases. These results imply that ceramides can affect lipid interactions also outside sphingomyelin-rich domains in biological membranes.

In conclusion, our study shows that ceramides have major impact of the lateral distribution of sterols in bilayer membranes, and that the effects also are reflected in the equilibrium distribution of e.g. sterols among different membranes. It is interesting to note that the most dramatic effects are induced by ceramides having physiologically relevant acyl chain compositions.

Acknowledgements

This study was supported by generous grants from the Academy of Finland, the Sigrid Juselius foundation, and the Åbo Akademi foundation.

References

[1] Y. Barenholz, Sphingomyelin–lecithin balance in membranes: composition, structure, and function relationships, in: M. Shinitzky (Ed.), Physiology of membrane fluidity, vol. 1, CRC press, Boca Raton, 1984, pp. 131–174.
[2] B. Ramstedt, J.P. Slotte, Membrane properties of sphingomyelins, FEBS Lett. 531 (2002) 33–37.
[3] J.P. Slotte, Cholesterol-sphingomyelin interactions in cells—effects on lipid metabolism, Subcell. Biochem. 28 (1997) 277–293.
[4] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.
[5] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) 31–39.
[6] D.A. Brown, E. London, Structure and function of sphingolipid- and cholesterol-rich membrane rafts, J. Biol. Chem. 275 (2000) 17221–17224.
[7] R.T. Dobrowsky, Sphingolipid signalling domains floating on rafts or buried in caves? Cell. Signal. 12 (2000) 81–90.
[8] C.J. Fielding, P.E. Fielding, Cholesterol and caveolae: structural and functional relationships, Biochim. Biophys. Acta 1529 (2000) 210–222.
[9] T. Harder, K. Simons, Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains, Curr. Opin. Cell Biol. 9 (1997) 534–542.
[10] S. Gatt, Studies of sphingomyelin and sphingomyelinases, Chem. Phys. Lipids 102 (1999) 45–53.
[11] Y.J.E. Björkqvist, T.K.M. Nyholm, J.P. Slotte, B. Ramstedt, Domain formation and stability in complex lipid bilayers as reported by cholestatrienol, Biophys. J. 88 (2005) 4054–4063.
[12] J. Sot, L.A. Bagatolli, F.M. Goni, A. Alonso, Detergent-resistant, ceramide-enriched domains in sphingomyelin/ceramide bilayers, Biophys. J. 90 (2006) 903–914.
[13] Ira, S. Zou, D.M. Ramirez, S. Vanderlip, W. Ogilvie, Z.J. Jakubek, L.J. Johnston, Enzymatic generation of ceramide induces membrane restructuring: correlated AFM and fluorescence imaging of supported bilayers, J. Struct. Biol. 168 (2009) 78–89.
[14] Ira, L.J. Johnston, Sphingomyelinase generation of ceramide promotes clustering of nanoscale domains in supported bilayer membranes, Biochim. Biophys. Acta 1778 (2008) 185–197.
[15] I. Johnston, L.J. Johnston, Ceramide promotes restructuring of model raft membranes, Langmuir 22 (2006) 11284–11289.
[16] Megha, E. London, Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function, J. Biol. Chem. 279 (2004) 9997–10004.
[17] J. Popov, D. Vobornik, O. Coban, E. Keating, D. Miller, J. Francis, N.O. Petersen, L.J. Johnston, Chemical mapping of ceramide distribution in sphingomyelin-rich domains in monolayers, Langmuir 24 (2008) 13502–13508.
[18] S.M. Alanko, K.K. Halling, S. Maunula, J.P. Slotte, B. Ramstedt, Displacement of sterols from sterol/sphingomyelin domains in fluid bilayer membranes by competing molecules, Biochim. Biophys. Acta 1715 (2005) 111–121.
[19] A.J. Guarino, S.P. Lee, S.P. Wrenn, Interactions between sphingomyelin and cholesterol in low density lipoproteins and model membranes, J. Colloid Interface Sci. 293 (2006) 203–212.
[20] C. Yu, M. Alterman, R.T. Dobrowsky, Ceramide displaces cholesterol from lipid rafts and decreases the association of the cholesterol binding protein caveolin-1, J. Lipid Res. 46 (2005) 1678–1691.
[21] S. Nybond, Y.J. Bjorkqvist, B. Ramstedt, J.P. Slotte, Acyl chain length affects ceramide action on sterol/sphingomyelin-rich domains, Biochim. Biophys. Acta 1718 (2005) 61–66.
[22] E.P. Kilsdonk, P.G. Yancey, G.W. Stoudt, F.W. Bangerter, W.J. Johnson, M.C. Phillips, G.H. Rothblat, Cellular cholesterol efflux mediated by cyclodextrins, J. Biol. Chem. 270 (1995) 17250–17256.
[23] A. Tsamaloukas, H. Szadkowska, H. Heerklotz, Thermodynamic comparison of the interactions of cholesterol with unsaturated phospholipid and sphingomyelins, Biophys. J. 90 (2006) 4479–4487.
[24] S.L. Niu, B.J. Litman, Determination of membrane cholesterol partition coefficient using a lipid vesicle-cyclodextrin binary system: effect of phospholipid acyl chain unsaturation and headgroup composition, Biophys. J. 83 (2002) 3408–3415.
[25] A. Tsamaloukas, H. Szadkowska, P.J. Slotte, H. Heerklotz, Interactions of cholesterol with lipid membranes and cyclodextrin characterized by calorimetry, Biophys. J. 89 (2005) 1109–1119.
[26] H. Ohvo, J.P. Slotte, Cyclodextrin-mediated removal of sterols from monolayers: effects of sterol structure and phospholipids on desorption rate, Biochemistry 35 (1996) 8018–8024.
[27] K.K. Halling, B. Ramstedt, J.H. Nystrom, J.P. Slotte, T.K. Nyholm, Cholesterol interactions with fluid-phase phospholipids: effect on the lateral organization of the bilayer, Biophys. J. 95 (2008) 3861–3871.
[28] R.T. Fischer, F.A. Stephenson, A. Shafiee, F. Schroeder, delta 5, 7, 9(11)-Cholestatrien-3 beta-ol: a fluorescent cholesterol analogue, Chem. Phys. Lipids 36 (1984) 1–14.
[29] L. Fugler, S. Clejan, R. Bittman, Movement of cholesterol between vesicles prepared with different phospholipids or sizes, J. Biol. Chem. 260 (1985) 4098–4102.
[30] R. Leventis, J.R. Silvius, Use of cyclodextrins to monitor transbilayer movement and differential lipid affinities of cholesterol, Biophys. J. 81 (2001) 2257–2267.
[31] J.R. Silvius, R. Leventis, Spontaneous interbilayer transfer of phospholipids: dependence on acyl chain composition, Biochemistry 32 (1993) 13318–13326.
[32] S.L. Veatch, S.L. Keller, Miscibility phase diagrams of giant vesicles containing sphingomyelin, Phys. Rev. Lett. 94 (2005) 148101.
[33] J. Sot, M. Ibarguren, J.V. Busto, L.R. Montes, F.M. Goni, A. Alonso, Cholesterol displacement by ceramide in sphingomyelin-containing liquid-ordered domains, and generation of gel regions in giant lipidic vesicles, FEBS Lett. 582 (2008) 3230–3236.
[34] Megha, P. Sawatzki, T. Kolter, R. Bittman, E. London, Effect of ceramide N-acyl chain and polar headgroup structure on the properties of ordered lipid domains (lipid rafts), Biochim. Biophys. Acta 1768 (2007) 2205–2212.
[35] Y. Lange, J. Ye, T.L. Steck, How cholesterol homeostasis is regulated by plasma membrane cholesterol in excess of phospholipids, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 11664–11667.
[36] A. Tsamaloukas, H. Szadkowska, H. Heerklotz, Thermodynamic comparison of the interactions of cholesterol with unsaturated phospholipid and sphingomyelins, Biophys. J. 90 (2006) 4479–4487.
[37] S.N. Pinto, L.C. Silva, R.F. de Almeida, M. Prieto, Membrane domain formation, interdigitation, and morphological alterations induced by the very long chain asymmetric C24:1 ceramide, Biophys. J. 95 (2008) 2867–2879.
[38] S. Chiantia, N. Kahya, P. Schwille, Raft domain reorganization driven by short- and long-chain ceramide: a combined AFM and FCS study, Langmuir 23 (2007) 7659–7665.
[39] M.R. Ali, K.H. Cheng, J. Huang, Ceramide drives cholesterol out of the ordered lipid bilayer phase into the crystal phase in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/cholesterol/ceramide ternary mixtures, Biochemistry 45 (2006) 12629–12638.
[40] J. Huang, G.W. Feigenson, A microscopic interaction model of maximum solubility of cholesterol in lipid bilayers, Biophys. J. 76 (1999) 2142–2157.
[41] J. Huang, J.T. Buboltz, G.W. Feigenson, Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers, Biochim. Biophys. Acta 1417 (1999) 89–100.
[42] B.M. Castro, L.C. Silva, A. Fedorov, R.F. de Almeida, M. Prieto, Cholesterol-rich fluid membranes solubilize ceramide domains: implications for the structure and dynamics of mammalian intracellular and plasma membranes, J. Biol. Chem. 284 (2009) 22978–22987.